CASSETTE FOR NUCLEIC ACID EXPRESSION IN PLANTS

The present invention relates to the expression of recombinant nucleic acids in plants. More specifically the invention provides a promoter for the selective expression of nucleic acids in stomatal guard cells, gene constructs containing said promoter, expression vectors carrying them and plants transfected therewith. The selective expression of nucleic acids in plant guard cells allows the regulation of stomatal opening/closing states thereby modulating, e.g. increasing, the plant ability to resist to adverse environmental or climatic conditions.

BACKGROUND OF THE INVENTION

Tissue-specific promoters for the generation of transgenic plants

The recent advancements in plant transformation techniques offer new opportunities to the improvement of crops. Following the transgenic approach, new characters can be introduced in the plants, which contribute to the increase of plant productivity, product quality and to improve the resistance of plants to adverse climatic conditions as well as to pathogens. In addition, transgenic plants can be used to produce recombinant proteins, biopolymers, medicaments, vaccines or antibodies (L. Lanfranco, Riv Biol. 2003, 96:31-54; Dunwell JM, J. Exp. Bot., 2000, 51:487-496).

The production of recombinant proteins in plants requires the use of promoters able to direct the correct expression of transgenes in vegetal tissues. To date, a limited number of promoters have been proposed for use in the generation of transgenic plants. Most of them are constitutive promoters, such as the 35S promoter from the cauliflower mosaic virus (Ca*MV*35S) (Odell et al., Nature, 1985, 313:810-812) or the ubiquitin promoter (Holtorf et al., Plant Mol. Biol., 1995, 29:637-646).

A drawback of such promoters is that they are active in nearly all the

plant tissues, thus preventing selective transgene expression in specific organs or during particular growth stages of the transgenic lineage, unlike tissue-specific promoters, which direct the production of recombinant proteins in selected tissues or organs. For example, the promoters involved in the accumulation of spare substances in seeds, such as phaseolina (Bustos et al., Plant Cell, 1989, 1:839-853) or 2S albumin (Joseffson et al., J. Biol. Chem., 1987, 262:12196-12201), direct the seed-specific expression of transgenes. The Rubisco small subunit promoter or the potato ST-LSI promoter direct leaf-specific transgene expression (Stockhouse et al., EMBO J., 1989, 8:2445). Although many other tissue- or organ-specific promoters have been described in the literature, only few of them show selectivity for a determined plant cell-type. These promoters should direct transgene expression limited to particular cells within the plant organ.

Stomata: anatomy and function

Stomata are small apertures present on the surface of aerial organs of land plants. These structures play an important role in the regulation of gas fluxes between the plant tissues and the atmosphere, allowing either CO_2 influx, which is necessary for the photosynthesis, or water loss by transpiration. The stoma consists of two highly specialized epidermal cells, called guard cells, the movement of which determines the opening/closure of the stomatal rima (FIGURE 1).

The level of stomatal opening reflects the balance between the need of CO_2 for the photosynthesis and water availability. Thus, it is not surprising that land plants have developed complex regulation mechanisms modulating the stomatal opening/closing process in response to environmental stimuli or to endogenous signals (Wilmer and Fricker, 1996, Stomata, Ed Chapman and Hall, London, 1-375).

The guard cell shape is determined by volume changes induced by

turgor modifications. The latter are in turn induced by the exchange of solutes, either inorganic, such as K^+ and Cl^-, or organic, such as saccharose or malate, in the cell lumen (Schroeder et al., Ann. Rev. Plant Physiol. Plant Mol. Biol., 2001, 52:627-6658). Conditions favouring the photosynthetic activity, such as the presence of light and of elevated CO_2 concentrations, promote the accumulation of solutes in the guard cells, whereby an increased turgor induces stomatal opening (FIGURE 1A).

On the contrary, in the absence of water, the phytohormon abscisic acid (ABA), induces a rapid diminution of guard cell turgor, resulting from the efflux of K^+, Cl^- and saccharose and from the conversion of malate into osmotic-inactive starch, thereby causing stomatal closure (FIGURE 1B).

The reduction of stomatal aperture, mediated by ABA accumulation, represents the main adaptive response of plants to drought, allowing to minimize the loss of water by transpiration (Wilkinson and Davies, Plant Cell Env., 2002, 25:195-210). Recently, many components of the ABA signal transduction cascade have been identified in guard cells following a pharmacological or genetic approach.

The ABA-induced stomatal closure involves the increase of Ca^{++} cytosolic concentration, the activation of anion channels, the modification of cytoplasmic pH and of potassium channel activity, the production of oxygen reactive molecules, the regulation of phosphatases and kinases and of other proteins such as heterotrimeric G-proteins, farnesyltransferase and mRNA cap-binding protein (Schroeder et al, Ann. Rev. Plant Physiol. Plant Mol. Biol., 2001, 52:627-6658).

The modulation of hormon signal transduction mechanisms, having a direct influence on stomatal opening/closure, provides a valuable tool for the generation of crop plants resistant to adverse environmental or climatic conditions, expecially to drought, and in which the exchange of CO_2, and

therefore the photosynthetic process, is optimized.

STATE OF THE ART

The modulation of ABA-induced signal transduction in the stomata enables the modification of the physiological response of guard cells to environmental stimuli. Many of the components involved in the mechanism of stomatal closure are known. Nonetheless, the modification of stomata activity is limited by the low availability of promoters specific for the guard cells.

Although a number of promoter sequences exerting their function in the guard cells have been described in the literature, none of them has shown sufficient selectivity. The promoters of *Arabidopsis* genes involved in the regulation of stomatal aperture, such as *Osm1* (Zhu et al., Plant Cell, 2002, 14:3009-3028), *Abh1* (Hugouvieux et al., Cell, 2001, 106:477-487), *Rac1* (Lemichez et al., Genes Dev., 2001, 15:1808-1816), *Kat1* (Anderson et al., Proc. Natl. Acd. Sci. U.S.A., 1992, 89:3736-3740), *Ost1* (Mustilli et al., Plant Cell, 2002, 14:3089-3099), *Chl1* (Guo et al., Plant Cell, 2001, 13:1761-1777), have showed activity not only in guard cells, but also in different cell types and plant organs. The lack of selectivity prevents the use of such promoters in the generation of transgenic plants with modified stomatal activity.

Besides modulating stomatal closure, ABA regulates many aspects of plant physiology and growth, including seed latency, the synthesis of storage proteins and lipids, phase transition and the response to wounds or pathogens (Finkelstein et al., Plant Cell, 2002, S15-S45).

The use of aspecific promoters for the ectopic expression of hormon signal transduction modulators may induce an alterated response in different plant tissues and organs, including stomatal guard cells. As a consequence, defects and abnormalities may arise, negatively affecting plant physiology, growth and productivity.

DESCRIPTION OF THE INVENTION

The invention provides a method for the selective expression of nucleic acids in plant stomatal guard cells by using promoter sequences of the *AtMYB60* gene (At1g08810; cDNA sequence deposited at GenBank acc. no. AF062895). In particular, the invention is based on the finding that different regions of *AtMYB60* promoter enable either ABA-responsive or ABA-independent selective expression of nucleic acids in stomatal guard cells.

According to a first embodiment, the invention provides a genetic construct or cassette for the selective expression of a nucleic acid sequence in plant stomatal guard cells, said construct or cassette containing the nucleic acid sequence functionally linked to the promoter sequence of *AtMYB60* gene (SEQ ID N. 1), or to fragments or variants thereof, said variants having at least 80%, preferably at least 90%, more preferably at least 95% sequence identity to SEQ ID No. 1, provided that said fragments or variants retain promoter activity on nucleic acid transcription.

According to preferred embodiments, the construct or cassette of the invention contains a fragment of the full-lenght *AtMYB60* promoter sequence (SEQ ID No. 1), which is selected from the group consisting of SEQ ID N. 2 (from nucleotide (nt) 1045 to 1291 of SEQ ID N. 1), SEQ ID N. 3 (nt 689-1291 of SEQ ID N. 1) and SEQ ID N. 4 (nt 293-1292 of SEQ ID N. 1).

Whereas the fragment extending from nt 1045 to nt 1291 of SEQ ID N. 1 exhibits an ABA-independent promoter activity, the activity of larger fragments, particularly those containing SEQ ID N. 3 and 4, as well as the activity of the full-lenght promoter (SEQ ID N. 1), are down-regulated by abscisic acid. Therefore, the stoma-specific expression of nucleic acids can be modulated in either ABA-dependent or ABA-independent manner using different gene constructs or expression cassettes according to the invention.

Besides the region of the *AtMYB60* gene endowed with transcription-

promoter activity, the expression cassette or constructs of the invention may contain genetic elements involved in transcription regulation, such as introns, polyadenylation sites at the gene 3'-end, transcription activators or enhancers, termination sequences, selection markers and leader sequences.

Any nucleic acid can be operatively linked to the *AtMYB60* promoter and inserted in the cassette or construct according to the invention. In particular, both coding and non-coding sequences can be used in the construction of the expression cassette. The encoded product, whether a peptide, protein or RNA transcript, is preferably involved in the intracellular signalling pathway modulated by abscisic acid (ABA) and in the cellular mechanisms regulating the stoma opening/closure state.

According to preferred embodiments of the invention, the *AtMYB60* promoter, fragments or variants thereof, are functionally linked to i) genes involved in the control of stomata aperture, in particular the *Osm1*, *Rac1*, *Kat1*, *Ost1* and *Chl1* genes (see above for the respective bibliographic references), ii) genes involved in the control of light-induced stomatal opening, particularly the guard cell blue-light photoreceptors *PHOT1* and *PHOT2* (Kinoshita T et al., Nature. 2001, 414:656-60), genes encoding for 14-3-3 proteins (Baunsgaard L et al., Plant J. 1998, 13:661-71), and the dual-affinity nitrate transporter gene *AtNRT1.1* (*CHL1*) (Guo FQ et al., Plant Cell. 2003, 15:107-17), iii) genes involved in the control of ABA-induced stomatal closure, particularly genes encoding for the following proteins: the type 2C protein phosphatases ABI, ABI2 and AtP2C-HA (Leung J et al., Plant Cell. 1997, 9:759-71.; Leonhardt N et al., Plant Cell. 2004, 16:596-615), the PP2A protein phosphatase RCN1 (Kwak JM et al., Plant Cell. 2002, 14:2849-61), the AAPK Ca^{2+}-independent protein kinase OST1 (Mustilli AC et al., J. Plant Cell. 2002, 14:3089-99), the SOS3-like calcium binding protein SCaBP5 and its interacting protein kinase PKS3 (Guo Y et al., Dev Cell. 2002, 3:233-

44), the AtrbohD and AtrbohF NADPH oxidases (Kwak, J. M. et al., EMBO J., 2003, 22:2623-33), the GTPase AtRac1 (Lemichez E et al., Genes Dev. 2001, 15:1808-16), the GTP-binding protein alpha subunit GPA1 (Wang XQ et al., Science. 2001, 29:292:2070-2), the syntaxin OSM1/SYP61 (Zhu, J. et al., Plant Cell, 2002, 14: 3009-28), the farnesyltransferase beta subunit ERA1 (Pei ZM et al., Science. 1998,282:287-90), the nitrate reductase NIA1 and NIA2 (Desikan R et al., Proc Natl Acad Sci U S A. 2002, 99:16314-8), K^+_{in} channels KAT1, KAT2, AKT2 (Kwak JM et al., Plant Physiol. 2001, 127:473-85), the K^+_{out} channels GORK (Hosy E et al., Proc Natl Acad Sci U S A. 2003, 100:5549-54), the nuclear RNA cap binding complex ABH1 subunit (Hugouvieux V et al., Cell. 2001, 106:477-87), the Sm-like snRNP protein SAD1 (Xiong L et al., Dev Cell. 2001, 1:771-81), and the the homeobox–leucine zipper transcription factor ATHB6 (Himmelbach A et al., Grill E., EMBO J. 2002, 21:3029-38).

Alternatively, nucleic acid sequences controlling the production of RNA transcripts exerting specific functions in the host cell, in particular antisense RNAs and siRNAs, can be inserted in the cassette or construct according to the invention.

As used herein, the expressions "funtionally linked" and "operatively linked" indicate that the promoter and nucleic acid making up the cassette or construct according to the invention, are in such a reciprocal orientation as to allow the promoter directing the expression of the nucleic acid, generally in 5'-3' orientation.

In a further aspect, the invention relates to expression vectors carrying the gene constructs or cassettes herein provided. The vectors can be bacterial plasmids, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), viral vectors, vectors for direct DNA transfer, or, preferably, vectors for *Agrobacterium*-mediated DNA transfer. The latter can

be either integrating or binary vectors and may contain selection markers, such as antibiotic- or herbicide-resistance genes, reporter genes facilitating the identification and selection of transformed cells, or sequences regulating gene expression in plants.

Direct transfer of DNA may include protoplast microinjection, electroporation and biolistic techniques based on plant bombardment with DNA-coated microparticles.

In a further aspect the invention provides transgenic plants, either monocotyledonous or dicotyledonous, as well as vegetative or reproductive parts thereof, or seeds, containing the genetic constructs according to the invention. In a preferred embodiment, the constructs or cassettes according to the invention are used to express proteins in the guard cell of closely related crop species, such as canola, in other dicotyledon plant, including soybean, tomato, tobacco, potato, cotton, or in monocotyledon species, such as corn, wheat, barley, rice.

The procedures for transforming plants with transgenic vectors or with naked DNA are known to those skilled in the art. For example, seeds at the germinative stage, seedlings or adult plants can be inoculated with *Agrobacterium* carrying the heterologous gene construct, and grown in suitable conditions.

The possibility of finely regulating stoma functions provides an important tool for the generation of plants able to efficiently respond to climatic changes. In particular, the possibility of inhibiting ABA-stimulated response thereby increasing the degree of stomatal opening and, consequently, the influx of CO_2 required for the photosynthetic process, is particularly important for plants cultivated in areas where water is not an environmental limiting factor. On the contrary, reducing stomatal aperture to avoid loss of water by transpiration is particularly beneficial to plants cultivated in dry

areas.

DETAILED DESCRIPTION OF THE INVENTION

Characterization of AtMYB60 promoter sequence

AtMYB60 is a member of the large family of R2-R3 MYB transcription factors of *Arabidopsis*. To examine the expression profile in wild-type *Arabidopsis* plants grown in standard conditions, different portions of the intergenic regions located either upstream of the translation initiation codon or downstream of the stop codon, were cloned upstream and, respectively, downstream the *GFP* (Green Fluorescence Protein) and GUS (β-glucuronidase) reporter genes (Figure 2B).

The constructs thus obtained were introduced in *Arabidopsis* and the resulting transgenic lines were histologically analyzed to detect the reporter expression domains. In all the samples examined, reporter gene expression was only detected in guard cells from all plant aerial organs provided with stomata (FIGURES 3-6).

Lines transformed with p1.3-2.2:GUS construct

The complementary and inverted sequence corresponding to the genomic region of Chromosome 1 comprised between nt 2821639 (3'UTR sequence of At1g08820 gene) and nt 2820349 (3'UTR sequence of *AtMYB60* gene -At1g08810) - according to the nomenclature used in "The Arabidopsis Information Resource" accessible at http://www.arabidopsis.org - was cloned upstream of the GUS reporter gene (FIGURE 2A, B, and FIGURE 7).

The intergenic region downstream of *AtMYB60* and comprised between the stop codon and the 5'UTR region of At1g08800 was inserted downstream of the same GUS reporter gene (FIGURE 2A, B). The genomic regions used in this construct contain the entire *AtMYB60* promoter and the putative regulatory elements located in the 3' region.

Subsequently, T2 plants obtained by transformation with the

p1.3-2.2:GUS construct were analyzed to determine the expression profile of the reporter gene. GUS staining was only found in stomatal guard cells from all the plant organs provided with such anatomical structures and at any growth stage (FUGURE 3). Hereinbelow, a detailed description of the results relating to different parts of the plant and to different growth stages are reported.

Seedling

Seedlings were analysed at days 4 and 7, i.e. at the stage of expanded cotyledon and at the moment of leaf appearance, respectively. A strong GUS-staining was observed at the level of stomatal guard cell, in both cotyledons and primary leaves, and in the hypocotyl (FIGURE 3). No staining was detected in the primary root and in its side branches.

Adult plant

The vegetative and reproductive organs were analysed in 7-week plants. GUS staining was present in stomatal guard cells from basal rosette leaves, cauline leaves and stems (FIGURE 4A, B, C).

As to the organs of the flower apparatus, GUS staining was detected in stomatal guard cells from sepals, pistils, anthers and in immature siliques (FIGURE 4D, E, F, G). The observation of petals, where stomata are absent, did not reveal any staining.

Lines transformed with p1.3:GUS, p0.9:GFP, p0.6:GUS, p0.2:GUS and p189:GUS constructs.

In order to confirm the results obtained from plants transformed with the p1.3-2.2:GUS construct and to delimit the genomic region necessary and sufficient for directing stam-specific expression of the reporter gene, the following constructs were prepared (FIGURE 2B):

- p1.3:GUS, containing the same intergenic region upstream of *AtMYB60* as that used in p1.3-2.2 GUS, cloned in front of the GUS reporter;

- p0.9:GFP, containing a 999bp genomic fragment upstream of *AtMYB60*, cloned in front of the GFP reporter (the activity of which can be detected by means of confocal microscopy);

- p0.6:GUS, containing a 603bp genomic fragment upstream of *AtMYB60*, cloned in front of the GUS reporter;

- p0.2:GUS, containing a 246bp genomic fragment upstream of *AtMYB60*, cloned in front of the GUS reporter;

- p189:GUS, containing a 189bp genomic fragment upstream of *AtMYB60*, cloned in front of the GUS reporter.

As shown in Figure 5, all the constructs analysed, with the only exception of p189:GUS, displayed the same expression profile as that obtained from plants transformed with p1.3-2.2:GUS construct. The presence of both reporter activities was observed solely in stomatal guard cells from all the seedling or plant structures provided with stomatal apertures.

In particular, the confocal-microscopy analysis of tissues from p0.9:GFP-transformed lines, clearly showed that the reporter expression was confined to stomatal guard cells, being the signal absent in any other cell-type (FIGURE 6).

ABA-induced modulation of reporter gene expression.

Recent studies have shown that the transcription regulation mediated by abscisic acid (ABA) represents an important control for stomatal physiological responses. Therefore, the effects of exogenous ABA administration on the expression of GUS and GFP reporter genes have been examined in the transgenic lines described above. The expression analysis was carried out with semiquantitative RT-PCR, and indicated that the levels of the GUS reporter transcript in p1.3-2.2:GUS. P1.3:GUS and p0.6:GUS lines, were significantly decreased by ABA administration (FIGURE 7). The same result was confirmed with the GFP reporter in p0.9:GFP lines. On the contrary, no

change in GUS expression was observed in p0:2GUS lines treated with ABA (FIGURE 7). These results indicate that the expression of genes fused to the promoter of *AtMYB60* (SEQ ID No. 1) is down-regulated by ABA. Further, the results indicated that the *cis* elements responsible for negative transcription modulation are contained between nucleotides -603 and -246, upstream of the translation initiation codon of *AtMYB60*.

Consequently, the entire promoter sequence SEQ ID No. 1, or fragments thereof containing the 246 bp portion upstream of the ATG codon, enable the ABA-independent expression of transgenes in stomatal guard cells.

DESCRIPTION OF THE FIGURES

Figure 1 - *Stomatal anatomy and function*

Optical microscopy photographies of *Arabidopsis* stomata present on leaf surface (bar= 5 μm). The stomata present on the epidermis of most of the aerial organs of soil plants are formed by two highly-specialized guard cells (g). Turgor changes in guard cells cause the aperture (panel A) or the closure (panel B) of the stomata rima.

Figure 2

(A) Schematic representation of the genomic region containing the *AtMYB60* gene. There are shown the end portion of the At1g08820-gene final exon, the three exons of the *AtMYB60* (At1g08810) coding region, and the initial portion of the At1g08800 gene - first exon.

(B) Schematic representation of constructs containing the GUS and GFP reporter genes under the control of different portions of the intergenic region between At1g08820 and *AtMYB60*. The p1.3-2.2:GUS construct further contains the entire intergenic region comprised between *AtMYB60* and At1g08800, inserted downstream of the GUS reporter. The length of each genomic region is indicated as number of bp.

Figure 3 - *Stoma-specific expression of the GUS reporter in seedlings*

from lines transformed with the p1.3-2.2:GUS construct

A) expression of the reporter GUS in seedlings at day 4. The staining is present only in stomatal guard cells in the cotyledons (c) and hypocotyl (i). No signal is found in the root (r).

B) cotyledon (particular)

C) leaf epidermis of a 7-day seedling (particular).

Figure 4 - *stoma-specific expression of the GUS reporter in adult plants from lines transformed with the p1.3-2.2:GUS construct*

A) expression of the reporter GUS in leaves from 7-week adult plants

B) leaf (particular)

C) stem (particular)

D) mature inflorescence: GUS staining is present only in the sepal stomata

E) mature flower: GUS staining is present only in sepal, anther and pistil stomata.

F) Pistil (particular)

G) Anther (particular)

Figure 5 - *expression of the GUS reporter in adult plants from lines transformed with p1.3:GUS, p0.6:GUS, p0.2:GUS and p189:GUS constructs*

Examples of staining of lines transformed with different constructs:

A) and B) 4-day seedling

C) rosette leaves

D) stem

F) mature flower

G) and H) rosette leaf stomata from plants transformed with the p189:GUS construct

Figure 6 - *stoma-specific expression of the GUS reporter in adult plants from lines transformed with the p0.9:GFP construct*

A) Expression of the GFP reporter in leaves from 7-week adult plants (bar = 1 μm)

B) Expression of the GFP reporter in a stem examined by confocal microscopy (bar = 20 μm)

C) Particular of a leaf stoma examined by confocal microscopy (bar = 2 μm)

Figure 7 - expression of the GUS and GFP reporter in plants treated with ABA

RT-PCR analysis of the GFP and GUS reporter expression in transgenic lines treated with 100 μM ABA for 6 hours. *TSB1* gene is used as the control.

MATERIALS AND METHODS

Plant growth

For in-plate growth, the seeds were sterilized as follows: 5 min in absolute ethanol, 5 min in 0.6% (v/v) sodium hypochlorite, 0.05% Tween 20, 2 washes in sterile water. The seeds were resuspended in 0.1% agarose sterile solution and germinated in Petri dishes containing 0.7% agarized MS medium (Sigma M-5519) added with 1% saccharose, pH 5.7. The plates were layered for 4 days at 4°C in the dark to allow uniform germination and then placed at 22°C with 16 hr light (48 $\mu E/m^2$) and 8 hr dark periods.

For the growth in soil, the seeds were layered at 4°C in the dark for a period of 4 days and then germinated in Einheitserde soil (VM-type, Manna-Italy) in Araflat plates (Arasystem, Betatech, Belgium) or in culture bottles, with a 16 hr light (48 $\mu E/m^2$) - 8 hr dark cycle.

Genomic DNA extraction

Seedlings that were grown in plates, as well as flowers or leaves from plants grown in soil, were placed in Eppendorf tubes and fozen in liquid nitrogen. The tissues were minced in the tubes, by means of a plastic tip fixed to a bench drill, in the presence of 500 μl extraction buffer (7M urea, 350 mM

Na_2SO_4, 50 mM Tris pH 8.0, 8 mM EDTA, 34 mM sarkosyl). The same volume of phenol and chloroform was then added (1:1 v:v) and, after vortexing, the samples were centrifuged at 13000 rpm for 5 min. The surnatant was placed in clean tubes and added with 400µl of distilled water and 0.7 volumes of isopropanol. The DNA was precipitated by centrifugation of the samples at 13000 rpm for 10 min. Isopropanol was removed and the pellet washed with 300 µl of 80% ethanol. After removal of ethanol, the DNA was resuspended in 40 µl of 50 mM Tris-HCl pH 8.0, 20 µg/ml of 5 mM EDTA. 2 µl of 20mg/ml Rnase A (Boehringer) were added and the samples were incubated at 37°C for 30 min. The extracted DNA was kept at -20°C.

Amplification of AtMYB60 5' and 3' genomic regions

The P60R5NEW primer (5' TCGGATCCTCTAGATCTCTCTG 3') was used for the amplification of different portions of the region upstream of the *AtMYB60* gene, in combination with the primers reported in the following Table. A BamHI restriction site (GGATCC) was introduced in the P60R5NEW primer.

Primer	Sequence 5'-3'	Region amplified with P60F1	Construct
P60F1	AAGCTTCACAAGGACACAAGGACA	1291bp	*p1.3:GUS p1.3-2.2:GUS*
P60F8	ATAGAATCTAACACTACTAATTGTTAT	999bp	*p0.9:GFP*
P60F2bis	AAGCTTCAAGTTGCAGTGAATGA	603bp	*p0.6:GUS*
P60F3	AAGCTTCGTGTGGAGATCAACAT	246bp	*p0.2:GUS*
P60F5	AAGCTTGCAGAGTGACTCGTGA	189 bp	*P189:GUS*

The AAGCTT sequence corresponding to a HindIII restriction site, was inserted to facilitate the cloning of genomic fragments.

The 3' genomic region, 2219bp in length, was amplified using the

primers 60-3'UTRF2 (5' CACTTGATGGAGCTCTCTAATATG 3') and 60-3' UTRR1 (5' CTGCAGACGTTTGTCTAGTAG 3').

The PCR reactions were carried out with 0.5 µg genomic DNA in a reaction mixture containing Red Taq PCR Reaction Buffer 1x (Sigma), dATP, dCTP, dGTP and dTTP (5 mM each), primers (25 µM each), 1 unit Red Taq™ polymerase (Sigma) and sterile distilled water to a final volume of 25 µl. The amplification reaction was performed as follows: 1 min at 94°C; 40 cycles at 94°C for 15 sec, 15 sec at the annealing temperature specific for the primer pair utilized, 72°C for 1 min; 72°C for 10 min. The reaction products were separated by electrophoresis on 1% (w/v) agarose gel in TBE 1X (89mM Tris-base, 89 mM H2BO3, 2 mM EDTA pH 8) and analysed with a UV transilluminator. The obtained bands were excised from the agarose gel and purified by means of Qiaquick Gel Extraction Kit (Quiagen), according to the manufacturer's instructions.

Preparation of the constructs containing the different genomic regions fused to the reporter gene

- p0.9:GFP construct

The 999bp genomic fragment was cloned in pCRII-TOPO vector (Invitrogen), according to the manufacturer's instructions. Subsequently, the fragment was excised by EcoRI cleavage and its ends were made non-sticky by treatment with Klenow (Roche), following the manufacturer's instructions. The fragment thus obtained was inserted in the binary vector pBIN mGFP-ER, containing the GFP reporter, previously digested with HindIII and treated with Klenow.

- p1.3-2.2:GUS construct

The 1291bp genomic fragment was cloned in the pCR4-TOPO vector (Invitrogen), following the manufacturer's instructions. This fragment was subsequently excised by HindIII, BamHI cleavage and cloned in the HindIII,

BamHI sites of the binary vector pBI101.3 (Stratagene), containing the GUS reporter, to produce the p1.3:GUS vector. The genomic fragment corresponding to the 3' region of AtMYB60, 2219bp in length, was inserted in pCRII-TOPO vector and subsequently excised by EcoRI cleavage. The EcoRI fragment was then inserted in the EcoRI site downstream the transcription terminus of the p1.3:GUS vector, to generate the p1.3-2.2:GUS vector.

- p0.6:GUS construct

The genomic fragment of 603bp was cloned in the pCR4-TOPO vector (Invitrogen), following the manufacturer's instructions. The fragment was subsequently excised by HindIII, BamHI cleavage and cloned in the HindIII, BamHI sites of vector pBI101.3 (Stratagene).

- p0.2:GUS construct

The 246bp genomic fragment was cloned in pCR4-TOPO vector (Invitrogen), following the manufacturer's indications. The fragment was subsequently excised by HindIII, BamHI cleavage and cloned in the HindIII, BamHI sites of the pBI101.3 binary vector (Stratagene).

- p189:GUS construct

The 189bp genomic fragment was cloned in pCR4-TOPO vector (Invitrogen), following the manufacturer's indications. The fragment was subsequently excised by HindIII, BamHI cleavage and cloned in the HindIII, BamHI sites of the pBI101.3 binary vector (Stratagene).

Plant transformation

Wild-type *Arabidopsis thaliana* plants belonging to the Columbia ecotype were grown at 22°C with a photoperiod of 16 hr ligth/ 8 hr dark. In order to increase seed production, the primary inflorescences were removed and the plants were grown for additional 5-6 days, until the secondary inflorescences appeared. All the siliques were eliminated prior to transformation. The plants were then transformed with the *Agrobacterium*

strain GV3101 by "floral-dip", following the Clough-Bent protocol (Clough and Bent, Plant J., 1998, 16:735-743).

Sterilized T1 seeds from transgenic plants were layered at 4°C in the dark for 4 days, and subsequently germinated in MS soil (Sigma M-5519), added with 0.8% bactoagar (Difco 0141-01) pH 5.7 and 100 μg/ml kanamycin. The plants were grown at 22°C, under 16hr light / 8hr dark photoperiod.

GUS assay

Seedlings, rosette and stem leaves, stalks, inflorescences and siliques were placed in microtiter plate wells containing 2.0 ml GUS-staining solution (100mM sodium phosphate pH 7.0, 0.1% Triton X-100, 1 mg/ml X-Gluc, 0.5 mM ferrocyanidine). The microtiter plate was placed in a vacuum-dryer for 10 min prior to incubation at 37°C for one night in the dark. The GUS-staining solution was then removed and the tissues were washed several times with absolute ethanol for 1 hr, until complete removal of staining. The tissues were kept at -20°C in 70% ethanol.

The reporter expression profiles were examined with the OLYMPUS SZX12 stereoscope (7X – 90X magnification).

Confocal microscope analysis

Samples for confocal microscope analysis were placed in a glass slide provided with a cover (COVERWELL PERFUSION CHAMBER OBLONG - Sigma), and immersed in a solution containing 0.3% gelrite, 1% saccharose and ½ MS pH 5.8 (GELRITE GELLANGUM Sigma). Subsequently, a histology slide was placed over.

The analysis was carried out with a TCS NT confocal microscope (LEICA) equipped with Argon-Krypton laser carrying a filter for GFP (488 nm excitation, 519 nm emission). Scanning was repeated several times at different magnification.

ABA treatment and RT-PCR analysis

The seeds from different transgenic lines were sterilized as described above and germinated in liquid MS soil containing 1% saccharose and 0.5 gL^{-1} MES. After three week growth under continuous shaking (120 rpm), ABA was added at a final concentration of 100 μM. The tissues for mRNA extraction were taken at time 0 and after 6 hr treatment. Total RNA was extracted by mincing the frozen tissues in 500 μl extraction buffer (1M Tris HCl pH 9, 20% Sodium Dodecyl Sulfate, 4M LiCl and 10mM EDTA).

After phenol chloroform extraction, the RNA was precipitated at 4°C in 4M LiCl, washed with 70% ethanol and resuspended in water treated with diethylpyrocarbonate (1‰ DEPC). 5μg total RNA were treated for 30 min with Dnase I (15 units - Boheringer Mannheim), following the manufacturer's protocol. The reverse-transcription reaction was performed with Reverse Transcriptase Superscript™ II (Life Technologies), according to the manufacturer's indications, using the oligo(dT) primer, formed by 17 dT residues and by the adapter 5'-GGGAATTCGTCGACAAGC-3'. The cDNA samples were amplified in a reaction mixture containing Red Taq PCR Reaction Buffer 1X (Sigma) and 5mM dATP, dCTP, dGTP and dTTP, 25 μM specific primers (Table below), 1 unit RED Taq™ polymerase (Sigma) and sterile distilled water to a final volume of 25 μl. The amplification was carried out under the following conditions: 1 min at 94°C; 20 cycles at 94°C for 15 sec, 60°C for 15 sec, 72°C for 1 min; 72°C for 10 min. The PCR products were separated on 1% agarose gel and transferred to Hybond N+ filters (Amersham) in 0.4N NaOH. Filters were hybridized with TSB1-, GUS- or GFP-specific probes amplified using the primers indicated in the Table below, and tagged with digoxigenin using the DIG-High Prime kit (Roche), following the manufacturer's instructions.

Primer	Gene	Sequence 5'-3'
TSFB1	TSB1	5'-CTCATGGCCGCCGGATCTTGA-3'
TSBR1	TSB1	5'-CTTGTCTCTCCATATCTTGAGCA-3'
GFPF1	GFP	5'-GGAGAAGAACTTTTCACTGGAGTTGTCCC-3'
GFPR1	GFP	5'-TAGTTCATCCATGCCATGTGTAATCCCAGC-3'
GUSF1	GUS	5'-AATAACGGTTCAGGCACAGC-3'
GUSR1	GUS	5'-CTGTGGAATTGATCAGCGTTG-3'